Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation

G van Loo[1], P Schotte[1], M van Gurp[1], H Demol[2],
B Hoorelbeke[2], K Gevaert[2], I Rodriguez[3], A Ruiz-Carrillo[4],
J Vandekerckhove[2], W Declercq[1], R Beyaert[1] and
P Vandenabeele*,[1]

[1] Flanders Interuniversity Institute for Biotechnology and Ghent University,
   Department of Molecular Biology, K.L. Ledeganckstraat 35, B-9000 Ghent,
   Belgium
[2] Flanders Interuniversity Institute for Biotechnology and Ghent University,
   Department of Medical Protein Research, K.L. Ledeganckstraat 35, B-9000
   Ghent, Belgium
[3] The Rockefeller University, Laboratory of Vertebrate Neurobiology, New York,
   USA
[4] Department of Molecular and Cell Biology, C.I.D., C.S.I.C., Biotechnology
   Reference Center of the Generalitat de Catalunya, Barcelona, Spain
* Corresponding author: K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium.
   Tel.: 32-9-264-51-31; Fax: 32-9-264-53-48;
   E-mail: peter.vandenabeele@rug.ac.be

Abstract

A hallmark of apoptosis is the fragmentation of nuclear DNA. Although this activity involves the caspase-3-dependent DNAse CAD (caspase-activated DNAse), evidence exists that DNA fragmentation can occur independently of caspase activity. Here we report on the ability of truncated Bid (tBid) to induce the release of a DNAse activity from mitochondria. This DNAse activity was identified by mass spectrometry as endonuclease G, an abundant 30 kDa protein released from mitochondria under apoptotic conditions. No tBid-induced endonuclease G release could be observed in mitochondria from Bcl-2-transgenic mice. The *in vivo* occurrence of endonuclease G release from mitochondria during apoptosis was confirmed in the liver from mice injected with agonistic anti-Fas antibody and is completely prevented in Bcl-2 transgenic mice. These data indicate that endonuclease G may be involved in CAD-independent DNA fragmentation during cell death pathways in which truncated Bid is generated. *Cell Death and Differentiation* (2001) **8,** 1136 – 1142.

Keywords: apoptosis; caspase; mitochondria; tBid; endonuclease G

Abbreviations: AIF, apoptosis inducing factor; CAD, caspase-activated DNAse; ICAD, inhibitor of caspase-activated DNAse; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PSD, post-source decay; tBid, truncated Bid

Introduction

Internucleosomal DNA fragmentation is a hallmark of the apoptotic process. The best characterized enzyme responsible for this DNA fragmentation is the caspase-activated DNAse CAD (also called DNA fragmentation Factor 40, DFF40)[1,2] that forms an inactive heterodimer with ICAD, the inhibitor of CAD (also called DFF45).[1,3] Under apoptotic conditions, ICAD is proteolyzed by caspase-3 causing the dissociation of the CAD/ICAD heterodimer, releasing CAD, which then translocates from the cytosol to the nucleus. Cells derived from mice that lack ICAD or that express caspase-resistant mutant ICAD have reduced DNA fragmentation, showing that CAD is the major DNAse implicated.[3,4] However, the development of these mice appeared to be normal, suggesting that CAD activity is not required for mammalian developmental cell death.[4,5]

Apoptosis Inducing Factor (AIF),[6] a mitochondrial flavoprotein translocated to the nucleus during the process of apoptosis is implicated in large scale ($\sim$50 kb) DNA fragmentation and peripheral chromatin condensation, but not oligonucleosomal DNA laddering.[7] Acinus, a chromatin condensation factor, was shown to induce nuclear pyknosis in the absence of DNAse activity.[8] Although no clear *in vivo* relevance has been provided yet, other factors being involved in nuclear DNA fragmentation during the apoptotic process have been described.[9,10]

In our search for proteins that are released from mitochondria after an apoptotic stimulus, we identified endonuclease G as such a candidate, using an *in vitro* reconstitution system with purified recombinant tBid or apoptotic cytosol from Fas-treated L929sAhFas cells on isolated mitochondria. Endonuclease G, a mitochondrial nuclease that has been suggested to play a role in mitochondrial DNA replication,[11] was clearly released together with cytochrome *c*. When incubated with purified nuclei, the tBid-induced mitochondrial supernatant could evoke DNA degradation. This activity was absent in supernatant of tBid-treated mitochondria from Bcl-2 transgenic mice and could not be prevented by caspase-inhibitors. The release of endonuclease G during apoptosis was confirmed *in vivo* in a model of anti-Fas induced fulminant hepatitis.

Results

tBid-induced release of mitochondrial proteins

To study the release of mitochondrial proteins by tBid during the process of apoptosis we generated recombinant murine tBid. Incubation of recombinant tBid with isolated murine liver mitochondria induced the release of cytochrome *c* (Figure
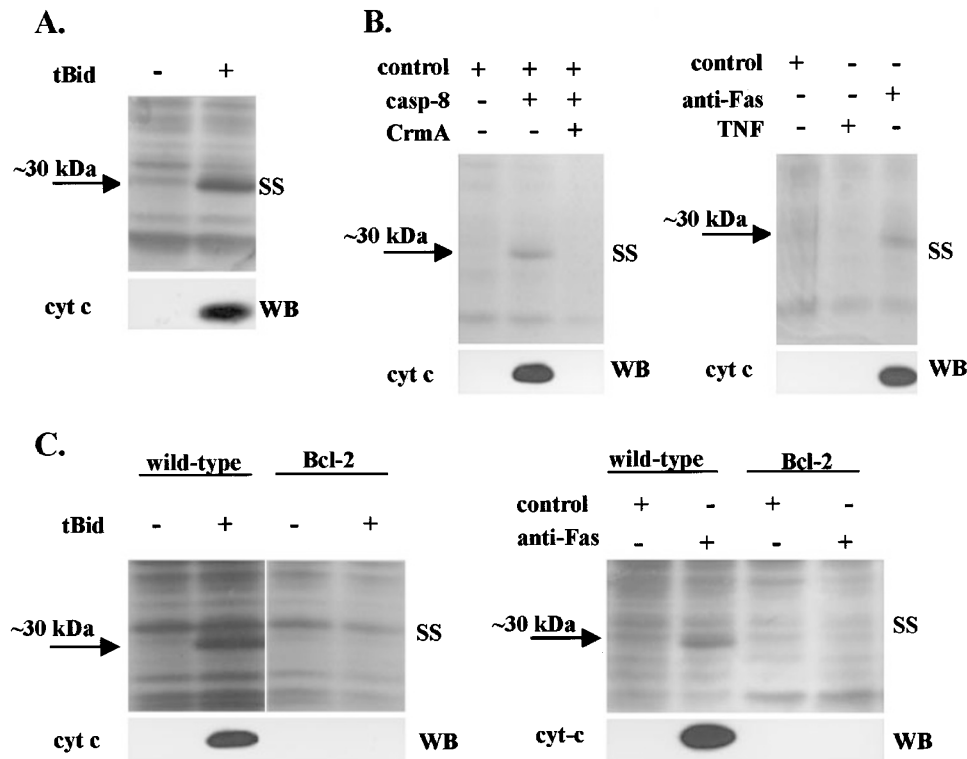
A.

B.

C.

Figure 1 Modulation of the release of a ~30 kDa protein in isolated mitochondria. (**A**) tBid-induced release of cytochrome *c* and a ~30 kDa protein. Mouse liver mitochondria were incubated with control buffer or 10 ng (corresponding to 6.7 nM) recombinant tBid. Supernatants were separated by means of 15% SDS–PAGE followed by Western Blotting (WB) using an anti-cytochrome *c* antibody or by silver staining (SS). (**B**) Induction of cytochrome *c* and the ~30 kDa protein release by apoptotic L929sAhFas cytosol. Non-treated L929sAhFas control cytosol was preincubated with recombinant murine caspase-8 (1 μg) and recombinant CrmA (5 μg) as indicated, and incubated with liver mitochondria (left panel). Non-treated L929sAhFas control cytosol, anti-Fas antibody-induced (250 ng/ml) L929sAhFas apoptotic cytosol and mTNF-induced (10 000 U/ml) L929sAhFas necrotic cytosol were incubated with liver mitochondria as indicated (right panel). The supernatants were recovered after centrifugation and immunoblotted with an anti-cytochrome *c* antibody (WB) or visualized by silver staining (SS). (**C**) Inhibition of tBid- and L929sAhFas cytosol-induced release of cytochrome *c* and ~30 kDa protein by Bcl-2. Recombinant tBid (10 ng) was incubated with liver mitochondria from wild-type or liver-specific Bcl-2 transgenic mice (left panel). Non-treated L929sAhFas control cytosol and anti-Fas antibody-induced (250 ng/ml) L929sAhFas apoptotic cytosol were incubated with liver mitochondria as indicated (right panel). The supernatants were recovered after centrifugation and immunoblotted with an anti-cytochrome *c* antibody (WB) or visualized by silver staining (SS)

1A). In parallel, a silver staining profile of the mitochondrial supernatant revealed the presence of a ~30 kDa protein as compared to untreated control supernatant. As endogenous Bid is cleaved under apoptotic conditions, we prepared cytosol from L929sAhFas cells and treated that with recombinant caspase-8. When incubated with purified mitochondria, this activated cytosol was able to induce the release of cytochrome *c* and the ~30 kDa protein, a process that was blocked in the presence of recombinant CrmA, a caspase-8 inhibitor (Figure. 1B, left panel). Neither untreated cytosol nor recombinant caspase alone was able to induce release of cytochrome *c* or the ~30 kDa protein (Figure 1B and data not shown). A similar observation was done using apoptotic cytosol from anti-Fas treated L929sAhFas cells (Figure 1B, right panel). Immunodepletion of Bid and tBid from the apoptotic cytosol abolished the capacity of the apoptotic cytosol to induce the release of cytochrome *c* and the ~30 kDa protein (data not shown and see Figure 3B), demonstrating that tBid is required. Under necrotic conditions however, using cytosol from TNF-treated L929sAhFas cells, no release of cytochrome *c* nor of the ~30 kDa protein could be observed (Figure 1B, right panel).

Inhibition of the ~30 kDa protein release by Bcl-2

To assess the question if Bcl-2 is able to prevent mitochondrial release of the ~30 kDa factor, we prepared liver mitochondria from wild-type and liver-specific Bcl-2 transgenic mice.[12] As expected, Bcl-2 blocked the release of cytochrome *c* induced by recombinant tBid (Figure 1C, left panel) and by apoptotic L929sAhFas cytosol (Figure 1C, right panel). Also the release of the ~30 kDa protein was abolished under these conditions as is appreciated from the silver staining profiles.

Mass spectrometric identification of the ~30 kDa factor as endonuclease G

In order to identify the ~30 kDa factor, a mitochondrial preparation from one mouse liver was exposed to 170 nM purified tBid and the mitochondrial supernatant was separated by means of 15% SDS–PAGE together with the supernatant of an untreated control, followed by Coomassie Brilliant Blue staining (Figure 2A). The ~30 kDa protein band, present in the tBid-induced supernatant, together with the corresponding
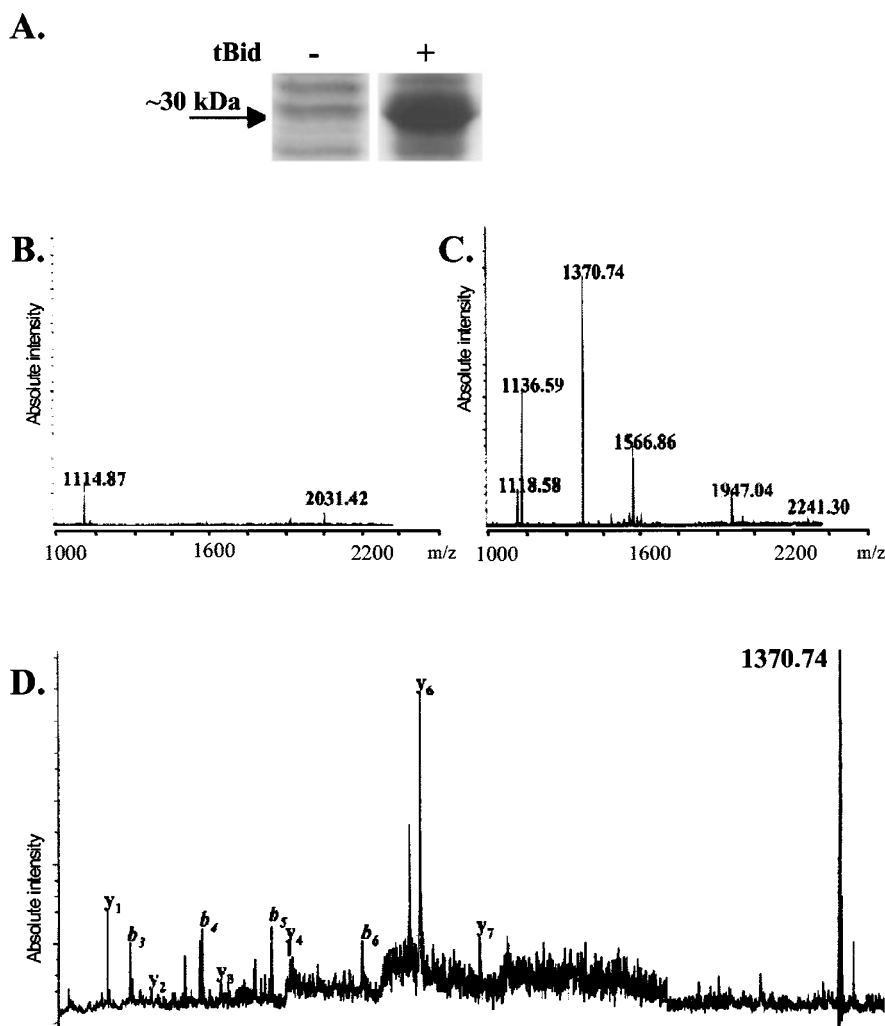
A.

B.

C.

D.

Figure 2 MALDI-MS identification of tBid-induced mitochondrial release of mouse endonuclease G. (**A**) A mitochondrial preparation of one liver was subjected to 15% SDS–PAGE and stained with Coomassie Brilliant Blue. (**B, C**) The ∼30 kDa protein band released by tBid and its control were digested using trypsin. The generated peptide mixture was separated by reverse phase-HPLC and shown are MALDI-MS spectra of peptides present in the HPLC-fraction from protein digests in the negative control (**B**) and tBid-released (**C**) proteins. (**D**) MALDI-PSD analysis of the peptide with a mass of 1370.74 Da present in the spectrum of B, led to the identification of the peptide baring the sequence NH_2-ASGLLFVPNILAR-COOH of the mouse endonuclease G protein

region of the lane loaded with control supernatant, were excised from the gel, trypsinized and used for MALDI-MS peptide mass fingerprint analysis.[13] MALDI-PSD analysis of a peptide with a mass of 1370.74 Da, present in the spectrum of the tBid-induced condition but absent in the negative control (Figure 2B and C), led to the identification of the peptide baring the sequence NH_2-ASGLLFVPNILAR-COOH from the mouse endonuclease G protein. This was further verified by manually checking the PSD-spectrum for the presence of other tryptic peptide fragments of endonuclease G (Figure 2D). Finally, we identified more fragments that cumulatively covered about 30% of the amino acid sequence of endonuclease G (data not shown). The identification was confirmed by Western blotting, demonstrating the clear tBid-induced release of the mitochondrial 30 kDa endonuclease G together with cytochrome *c* (Figure 3A). Immunodepletion of endogenous Bid from apoptotic cytosol completely blocked the capacity of apoptotic cytosol to induce the release of

endonuclease G (Figure 3B) indicating that Bid is sufficient and essential for mitochondrial endonuclease G release.

Endonuclease G allows caspase-independent nuclear DNA degradation during apoptosis

Endonuclease G is a mitochondrial nuclease that has been suggested to play a role in mitochondrial DNA replication[11] but a role in nuclear DNA fragmentation or an involvement in the apoptotic process was never shown, until recently.[14,15] As endonuclease G was identified in the mitochondrial super-natant that is released under apoptotic conditions, we investigated the hypothesis that the tBid-induced mitochon-drial supernatant possessed intrinsic DNAse activity on isolated nuclei as compared to control supernatant. U937 nuclei were isolated and incubated with supernatant of untreated or tBid-treated mitochondria. As shown in Figure 4A, tBid-induced mitochondrial supernatant is able to induce
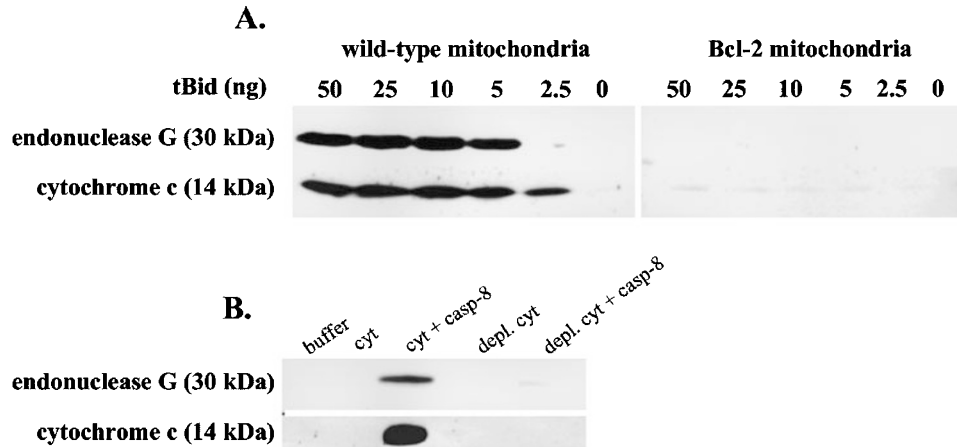
A.

	wild-type mitochondria						Bcl-2 mitochondria					
tBid (ng)	50	25	10	5	2.5	0	50	25	10	5	2.5	0
endonuclease G (30 kDa)												
cytochrome c (14 kDa)												

B.

	buffer	cyt	cyt + casp-8	depl. cyt	depl. cyt + casp-8
endonuclease G (30 kDa)					
cytochrome c (14 kDa)					

Figure 3 tBid is sufficient and required for mitochondrial endonuclease G release. (**A**) tBid was incubated with purified liver mitochondria from wild-type and Bcl-2 transgenic mice. Supernatants were separated from the mitochondrial pellets by centrifugation and subjected to 15% SDS – PAGE, followed by immunoblotting with anti-endonuclease G and anti-cytochrome c antibody. (**B**) Endogenous Bid was immunodepleted from L929sAhFas cytosol (cyt) activated with recombinant caspase-8 (1 μg) prior to incubation with mitochondria, as indicated. Supernatants were isolated and subjected to SDS-PAGE followed by immunoblotting with anti-endonuclease G or anti-cytochrome c
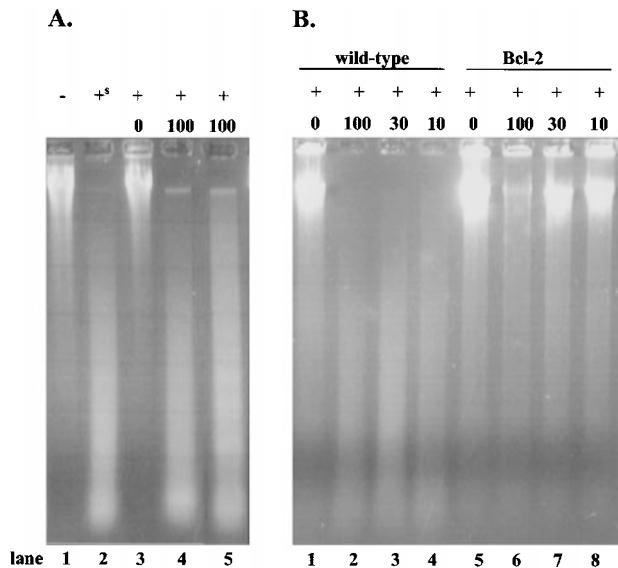
A.

-	+^{s}	+	+	+
		0	100	100

| lane | 1 | 2 | 3 | 4 | 5 |

B.

	wild-type				Bcl-2			
+	+	+	+	+	+	+	+
0	100	30	10	0	100	30	10

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

Figure 4 tBid mitochondrial supernatant induces DNA fragmentation on isolated U937 nuclei, an activity that is absent in Bcl-2 mitochondrial supernatant. (**A**) U937 nuclei were incubated with supernatant of wild-type mitochondria induced with 0 or 100 ng tBid (lanes 3 and 4 – 5 respectively) in the absence (lane 1 – 4) or presence of DEVD-fmk inhibitor (lane 5). As controls, U937 nuclei were incubated with buffer alone (lane 1) and sonicated mitochondria (lane 2). (**B**) U937 nuclei were incubated with supernatant of wild type mitochondria (lanes 1 – 4) or Bcl-2 transgenic mitochondria (lane 5 – 8) induced with 0, 100, 30, and 10 ng tBid respectively. All samples (**A** and **B**) were analyzed by means of agarose gel electrophoresis and detection of DNA with ethidium bromide staining

DNA fragmentation while control supernatant is not. In Bcl-2 mitochondria no DNAse activity is released when exposed to tBid (Figure 4B). This observation is in accordance with the fact that endonuclease G release is prevented in mitochondria from Bcl-2 transgenic mice (Figure 1C). Western blotting revealed no caspase immunoreactivity in the supernatant of the mitochondria (caspases-1, -2, -3, -6, -7, -8, -9, -11; data

not shown). In line with that, no DEVDase activity was detectable in tBid-induced mitochondrial supernatant (data not shown) nor could the caspase-3 specific inhibitor DEVD-fmk block the DNA fragmentation generated by tBid-induced mitochondrial supernatant (Figure 4A). These data clearly show that the nuclease activity on isolated nuclei acts independent of caspase activity and is different from the caspase-3 dependent nuclease activity of CAD.

To verify the *in vivo* relevance of the release of endonuclease G during the apoptotic process, C57BL/6 wild-type and Bcl-2 transgenic mice[12] were injected with agonistic anti-Fas antibody. While Bcl-2 transgenic mice were very well protected against a lethal dose of anti-Fas antibody, wild-type mice died of massive liver apoptosis (data not shown). As shown in Figure 5, release of endonuclease G from mitochondria to cytosol, together with cytochrome *c*, can be demonstrated in anti-Fas-injected wild-type mice, in contrast to Bcl-2 littermates.

These data indicate that endonuclease G also *in vivo* is released during the apoptotic process and may participate in DNA degradation.

Discussion

In our search for proteins that are released from mitochondria during the process of apoptosis, we used an *in vitro* reconstitution system in which isolated mitochondria were treated with purified recombinant tBid or endogeneous tBid using apoptotic cytosol from anti-Fas antibody treated L929sAhFas cells (Van Loo *et al.*, submitted). One of the proteins we identified by mass spectrometric analysis of the mitochondrial supernatant was endonuclease G. Endogenous levels of Bid are sufficient and essential for endonuclease G release from mitochondria as immunodepletion of Bid from cytosol incubated with mitochondria *in vitro* completely abrogated the release of the protein. Apoptotic release of mitochondrial endonuclease G was completely blocked in conditions where Bcl-2 was overexpressed. Under necrotic
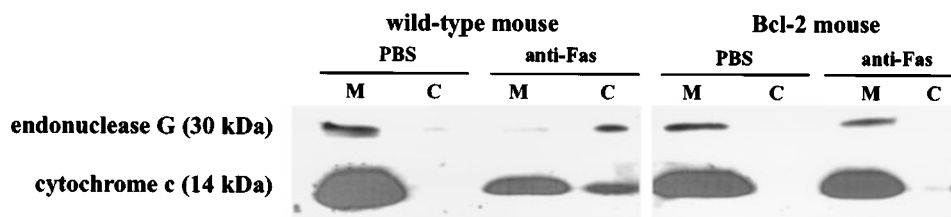
Figure 5 Endonuclease G is released from mitochondria of apoptotic cells during *in vivo* apoptosis. Wild type and Bcl-2 transgenic mice were intravenally injected with PBS or agonistic anti-Fas antibody and liver mitochondria were prepared as described in Materials and Methods. One hundred μg of mitochondrial (M) and cytosolic (C) lysates were subjected to 15% SDS–PAGE, followed by immunoblotting with anti-endonuclease G or anti-cytochrome *c* antibody

conditions, endonuclease G seems not to be released from mitochondria as was shown with necrotic L929sAhFas cytosol reconstituted with purified mitochondria *in vitro*.

Endonuclease G is a mitochondrial nuclease that is likely implicated in mitochondrial DNA replication[11] and is synthesized as a propeptide with an amino-terminal presequence that targets the nuclease to mitochondria.[11] Besides these data, little is known about the precise function of endonuclease G but it was never associated with nuclear DNA fragmentation or cell death in general. Although endonuclease G implication in mitochondrial DNA replication favors the idea of a matrix localization, the released mitochondrial fraction of endonuclease G is rather confined to the intermembrane space as we could clearly show that the matrix protein adenylate kinase 3 was retained within the mitochondria after exposure to tBid (data not shown; Van Loo *et al.*, submitted).

As we identified endonuclease G as one of the proteins that is clearly released from mitochondria by an apoptotic trigger, we addressed the question if this nuclease could be involved in apoptotic nuclear DNA fragmentation. Our experiments revealed that the mitochondrial supernatant that was released under apoptotic conditions with tBid could clearly induce DNA fragmentation when incubated with isolated nuclei. This activity was absent in tBid-induced mitochondrial supernatant from Bcl-2 transgenic mice. Moreover, neither procaspases or activated caspases were detectable by Western blotting or by an enzymatic assay, nor did the addition of caspase-inhibitors affect nuclear DNA degradation, demonstrating a caspase-independent action of this DNAse activity. The *in vitro* data obtained with isolated mitochondria could be confirmed *in vivo* in a murine model of lethal hepatitis. Administration of agonistic anti-Fas antibodies induced the release of both endonuclease G and cytochrome *c* from the mitochondria to the cytosol. This mitochondrial release was completely absent in Bcl-2 transgenic mice. Since CAD and ICAD are very low expressed in liver tissue ([5,16] and S Nagata, personal communication), the clear DNA fragmentation observed in this tissue during fulminant hepatitis may be mediated by mitochondrial endonuclease G release. However, the actual contribution of this nuclease in apoptotic DNA fragmentation remains to be determined.

These data open an interesting possibility of defining a caspase-independent cell death pathway leading to DNA fragmentation. Many different apoptogenic stimuli impinge on the specific proteolysis of Bid. Proteolysis of Bid is exerted by caspases to generate 15 kDa tBid,[17,18] by

granzyme B to generate 14 kDa gtBid[19] or by lysosomal proteases.[20] The latter two proteolytic cascades may allow the release of endonuclease G independent of active caspases resulting in caspase-independent DNA degradation. A DNAse that can act independently of caspases may still allow DNA fragmentation in the presence of exogenous or endogenous caspase inhibitors.[21] This caspase-independent DNA fragmentation also impairs the definition of apoptosis as a caspase-dependent phenomenon.[21]

Recently, Li *et al.*[15] encountered the same caspase-independent nuclease pathway initiated from mitochondria. The residual nucleosomal DNA fragmentation observed in ICAD transgenic mice and in caspase-resistant ICAD mice,[4,5] could be due to endonuclease G, as the release and translocation of endonuclease G from mitochondria to the nucleus in ICAD-deficient embryonic fibroblasts has been observed. In another study, using a functional genetic screen, the nuclease *csp-6* was identified as the first mitochondrial protein involved in programmed cell death in *C. elegans. Csp-6* is most probably the nematode counterpart of endonuclease G[14] providing evidence for an evolutionarily conserved link between the mitochondria and nuclear apoptosis.

To conclude, these data suggest that the tBid-induced release of endonuclease G may be involved in CAD-independent DNA fragmentation. Under these conditions caspase-3 would not be required for nuclear DNA degradation.

Materials and Methods

Reagents

Monoclonal hamster anti-mouse Fas antibody Jo2 (Pharmingen, San Diego, CA, USA) was dissolved in endotoxin-free PBS and injected intravenally (15 μg/200 μl per mouse). Livers were excised from sacrificed mice and placed into ice-cold homogenization buffer (5 mM KH_2PO_4 Ph 7.4, 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS). The caspase peptide inhibitor acetyl-Asp-Glu-Val-Asp-fluoromethylketone (Ac-DEVD-fmk) was obtained from Enzyme Systems Products (Dublin, CA, USA), and the caspase fluorogenic substrate acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-amc) was obtained from Peptide Institute Inc. (Osaka, Japan).

Expression and purification of recombinant proteins

Murine tBID (residues 60–195) with a tag of six histidines at the N-terminus was cloned in the pLT10 vector and expressed in *Escherichia coli*. The protein was recovered in the soluble bacterial fraction and

purified by cobalt-immobilized metal affinity chromatography (TALON, Clontech, Palo Alto, CA, USA) using the manufacturer's protocol. Active recombinant murine caspase-8 was expressed and purified by a similar method.[22] Recombinant cowpox CrmA protein was a generous gift from Dr. G Salvesen (Burnham Institute, La Jolla, CA, USA).

Isolation of murine liver mitochondria

Livers of C57BL/6 wild-type and bcl-2 transgenic mice[12] were homogenized using a Wheaton type B douncer in homogenization buffer (5 mM KH_2PO_4 Ph 7.4, 0.3 M sucrose, 1 mM EGTA, 5 mM 3-(N-morpholino)propanesulfonic acid). The homogenates were cleared by centrifugation at $1800 \times g$ for 10 min at 4°C to remove intact cells and nuclei. The supernatant was spun down at $10\,000 \times g$ for 10 min at 4°C to precipitate the mitochondria containing heavy membrane fraction and further purified on a Percoll gradient as described earlier.[23] The mitochondrial pellet was resuspended in cell free system (CFS) buffer (10 mM HEPES-NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$ 5 mM pyruvate, 0.1 mM PMSF, 1 mM dithiothreitol), kept on ice and used within 1 h of preparation.

Western Blot analysis of tBID induced release of mitochondrial proteins

Intact mouse liver mitochondria equivalent of 40 μg protein were incubated at 37°C in 100 μl CFS buffer for 20 min with various reagents as indicated in the figure legends. The supernatants were separated from the mitochondria by centrifugation at $20\,000 \times g$ for 10 min at 4°C. One fifth of the supernatant was subjected to 15% SDS–PAGE followed by Western Blotting with anti-cytochrome c antibody (clone 7H8.2C12, Pharmingen, CA, USA) or anti-endonuclease G antibody which was made against the peptide AMDDT-FYLSN. Blots were visualized with the chemiluminescence NEN Renaissance method (Du Pont, Wilmington, DE, USA) after incubation of membranes with secondary antibody coupled to horseradish peroxidase.

Preparation of L929sAhFas lysates

L929sAhFas cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), L-glutamine (0.03%) and fetal calf serum (10%). Cells were left untreated, stimulated with 250 ng/ml agonistic anti-human Fas antibody (clone 2R2, Cell Diagnostica, Müenster, Germany) for 30 min to 2 h or with 10 000 U/ml recombinant murine TNF (produced in our laboratory by Alex Raeymaeckers) for 2–7 h. Cells were washed in cold PBS and permeabilized in 0.02% digitonin (Boehringer Mannheim GmbH, Mannheim, Germany) dissolved in CFS buffer and left on ice for 1 min. This treatment allows selective lysis of the outer cell membrane without affecting the organelle membranes. Lysates were cleared by centrifugation at $20\,000 \times g$ for 10 min and stored at 4°C. For Bid depletion experiments, the digitonin lysate of L929sAhFas cells was precleared three times by incubation for 5 min with 20% protein G beads (Amersham Pharmacia Biotech, Rainham, UK) and incubated with anti-Bid antibody (10 μg/ 5×10^6 cells) (RD Systems Inc., Minneapolis, MN, USA) and the mixture is rocked in the cold for 1.5 h at 4°C. The antibody complexes were captured with protein G beads (20%) for 30 min at 4°C and removed by centrifugation. The incubation with anti-Bid antibody was repeated. The resulting Bid-depleted supernatant was used in the reconstitution experiment with purified liver mitochondria.

Protein identification using MALDI-MS

For purification purposes, a mitochondrial preparation from one liver (corresponding to approximately 1 mg total protein) was incubated with 170 nM purified tBid for 20 min at 37°C (500 ng tBid/200 μl of mitochondria equivalent to 1 mg of total protein). Supernatant was removed from the mitochondria by centrifugation at $20\,000 \times g$ for 10 min at 4°C. Proteins were separated by 15% SDS–PAGE followed by Coomassie Brilliant Blue staining. The $\sim$30 kDa protein band in the tBid-induced mitochondrial supernatant and the corresponding bands at the same height in the supernatant of unstimulated mitochondria, were excised and in gel digested using trypsin, as described previously.[26] Following digestion, the supernatant containing the tryptic peptides was removed from the gel pieces and acidified using 1 μl of formic acid. A small fraction (10%) of this mixture was concentrated on Poros 50 R2 beads (Roche Molecular Biochemicals, Basel, Switzerland) and used for MALDI-MS peptide mass-fingerprint analysis, as described previously.[13] Since the excised gel bands contained multiple proteins, no unambiguous protein identification could be made by solely using the obtained tryptic peptide mass maps. Therefore the remainder of the peptide mixture was loaded on a 1 mm i.d. $\times 50$ mm Vydac C18-column (Vydac Separations Group, Hesperia, CA, USA) and the peptides were separated by reverse phase-HPLC using an acetonitrile gradient. Eluting peptides were automatically collected in an acqueous solution containing a small number of Poros 50 R2 beads.[13] These fractions were completely dried in a centrifugal vacuum concentrator and stored at -20°C until further analysis by MALDI-MS. All MALDI-MS experiments were performed on a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The peptides present in each reverse phase-HPLC fraction were first scanned in reflectron mode and peptides that were clearly enriched compared to the negative control sample were further on selected for MALDI-PSD analysis. The peptide fragmentation spectra obtained were automatically used for protein identification in a nonredundant protein database using the MASCOT-algorithm (http://www.matrixsciences.com).

Isolation of U937 nuclei and measurement of DNA fragmentation

Exponentially growing U937 cells (200×10^6 cells) were incubated for 30 min with 10 μM cytochalasin B (Sigma, St. Louis, MO, USA) and collected by centrifugation for 5 min at $200 \times g$. Cells were washed in cold PBS and rocked on ice for 20 min in NB buffer (10 mM HEPES-NaOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF). Cells were dounced 20 times and the homogenate was brought over a double volume of a sucrose cushion in NB complete buffer (NB+30% sucrose) and spun down at $500 \times g$ for 6 min at 4°C. The nuclear pellet was washed in CFS and nuclei were resuspended in CFS buffer. For studying DNA fragmentation, 10^6 U937 nuclei were incubated in CFS buffer with a quarter of the mitochondrial supernatant for 2 h at 37°C in a total volume of 30 μl. Nuclei were lysed in TE buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA) with 0.5% SDS and 0.5 mg/ml proteinase K for 4 h at 37°C. Proteins were extracted with TE-buffered phenol and DNA was precipitated with two volumes EtOH and 1/10 volume of 4 M NaCl by overnight incubation at -20°C. The DNA pellet was resuspended in $0.1 \times$ TE and loaded on a horizontal 1.8% agarose gel.

Acknowledgements

References

1. Liu X, Zou H, Slaughter C and Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89: 175–184
2. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391: 43–50
3. Sakahira H, Enari M and Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391: 96–99
4. Zhang J, Liu X, Scherer DC, van Kaer L, Wang X and Xu M (1998) Resistance to DNA fragmentation and chromatin condensation in mice lacking the DNA fragmentation factor 45. Proc. Natl. Acad. Sci. USA 95: 12480–12485
5. McIlroy D, Tanaka M, Sakahira H, Fukuyama H, Suzuki M, Yamamura K, Ohsawa Y, Uchiyama Y and Nagata S (2000) An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. Genes Dev. 14: 549–558
6. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Constantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM and Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397: 441–446
7. Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, Costantini P, Ferri KF, Irinopoulou T, Prevost MC, Brothers G, Mak TW, Penninger J, Earnshaw WC and Kroemer G (2000) Two Distinct Pathways Leading to Nuclear Apoptosis. J. Exp. Med. 192: 571–580
8. Sahara S, Aoto M, Eguchi Y, Imamoto N, Yoneda Y and Tsujimoto Y (1999) Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature 401: 168–173
9. Robertson JD, Orrenius S and Zhivotovsky B (2000) Review: nuclear events in apoptosis. J. Struct. Biol. 129: 346–358
10. Los M, Neubuser D, Coy JF, Mozoluk M, Poustka A and Schulze-Osthoff K (2000) Functional characterization of DNase X, a novel endonuclease expressed in muscle cells. Biochemistry 39: 7365–7373
11. Cote J and Ruiz-Carrillo A (1993) Primers for mitochondrial DNA replication generated by endonuclease G. Science 261: 765–769
12. Rodriguez I, Matsuura K, Khatib K, Reed JC, Nagata S and Vassalli P (1996) A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection. J. Exp. Med. 183: 1031–1036
13. Gevaert K, Demol H, Sklyarova T, Vandekerckhove J and Houthaeve T (1998) A peptide concentration and purification method for protein characterization in the subpicomole range using matrix assisted laser desorption/ionization-post-source decay (MALDI-PSD) sequencing. Electrophoresis 19: 909–917
14. Parrish J, Li LY, Klotz K, Ledwich D, Wang X and Xue D (2001) Mitochondrial endonuclease G is important for apoptosis in *C. elegans*. Nature 412: 90–95
15. Li LY, Luo X and Wang X (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412: 95–99
16. Kawane K, Fukuyama H, Adachi M, Sakahira H, Copeland NG, Gilbert DJ, Jenkin NA and Nagata S (1999) Structure and promoter analysis of murine CAD and ICAD genes. Cell Death Differ. 6: 745–752
17. Li H, Zhu H, Xu CJ and Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94: 491–501
18. Luo X, Budihardjo I, Zou H, Slaughter C and Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481–490
19. Heibein JA, Goping IS, Barry M, Pinkoski MJ, Shore GC, Green DR and Bleackley RC (2000) Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. J. Exp. Med. 192: 1391–1402
20. Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, Ellerby LM, Bredesen D, Freeze H, Abrahamson M, Bromme D, Krajewski S, Reed JC, Yin XM, Turk V and Salvesen GS (2001) Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J. Biol. Chem. 276: 3149–3157
21. Borner C and Monney L (1999) Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ. 6: 497–507
22. Van de Craen M, Van Loo G, Declercq W, Schotte P, Van den brande I, Mandruzzato S, van der Bruggen P, Fiers W and Vandenabeele P (1998) Molecular cloning and identification of murine caspase-8. J. Mol. Biol. 284: 1017–1026
23. Schotte P, Van Criekinge W, Van de Craen M, Van Loo G, Desmedt M, Grooten J, Cornelissen M, De Ridder L, Vandekerckhove J, Fiers W, Vandenabeele P and Beyaert R (1998) Cathepsin B-mediated activation of the proinflammatory caspase-11. Biochem. Biophys. Res. Commun. 251: 379–387